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Actions of intrathecal diphtheria toxin-substance P fusion protein on models of persistent pain

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Abstract

Substance P (SP) plays a central role in the transduction of second messenger signals from primary afferent nociceptive terminals to second-order neurons in the spinal cord. We have tested a recombinant engineered diphtheria toxin/SP fusion protein (DAB389SP) in acute and chronic pain models in the rat. DAB389SP binds to the SP receptor (SPR) and is internalized and kills SPR-expressing cells by blocking cellular protein synthesis. DAB389SP delivery was by intrathecal infusion, of varying duration, at the lumbar level. In the chronic constriction injury model of neuropathic pain a significant reduction in mechanically induced hyperalgesia was obtained. This effect was less marked in an acute carageenan inflammation model. Although other pain characteristics (mechano-allodynia, cold-allodynia, and heat-hyperalgesia) showed some improvement, these were less pronounced. Immunocytochemistry revealed a toxin-induced reduction in lamina I, of SPR and of NMDA NR1 subunit receptor expressing neurons, and of c-Fos, an inducible molecular marker of persistent nociceptive activity. The use of cytotoxic fusion proteins to target specific cell types may be of considerable benefit in the study of nociception and the treatment of chronic pain.

Keywords: Cytotoxins; Immunotoxins; Substance P

1. Introduction

Substance P (SP) is an 11 amino acid peptide present in neuronal systems throughout the central nervous system (CNS). Direct tests of neuronal excitability demonstrate that SP is a potent neuroactive peptide that acts primarily on SP receptor (SPR)-expressing neurons. This receptor, classified as the neurokinin 1 (usually abbreviated as NK1) receptor, is a member of the seven transmembrane G-protein coupled receptor superfamily (Watling et al., 1995). Based on anatomical, electrophysiological, and pharmacological studies, SP has been shown to be involved in diverse motor, autonomic and sensory system functions. Although the exact role that SP and its receptor play in signaling nociception is unclear, they are particularly abundant in lamina I of the spinal cord (Brown et al., 1995). Moreover, electrical stimulation of peripheral nociceptors at C-fiber intensity induces the release of SP in the spinal cord (Duggan et al., 1988). When SP binds to its receptor, one or more cascades of second messenger signaling pathways are activated (see review by Krause et al., 1993) eventually triggering endocytosis of the SP/SPR complex. Within the endosome SP is degraded, and the SPR recycles to the surface (Grady et al., 1995). This SP/SPR internaliza-

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tion has been shown in epithelial cells and striatal neurons exposed to SP and in spinal cord neurons after somatosensory stimulation (Garland et al., 1994; Mantyh et al., 1995a; Mantyh et al., 1995b). Ligand-receptor endocytosis provides a route for a targeted delivery of a wide range of experimental and therapeutic molecules (Kato and Sugiyama, 1997).

Recombinant toxins as therapeutic agents have been thoroughly evaluated in medicine. Immunotoxins, generally conjugates of a cytotoxin and an antibody that recognizes a cell-surface protein, have been extensively used in the treatment of malignancies at peripheral and CNS sites (Pastan et al., 1992; Leppla, 1995; Sweeney and Murphy, 1995; Wiley, 1996; Youle, 1996). In a similar fashion, ligands have been placed on toxins to direct them at cell surface receptors. A carboxy-terminal truncated diphtheria toxin, that retains its transmembrane and catalytic domains but in which SP replaced the native binding region, was produced by recombinant technology (Fisher et al., 1996). This fusion protein (DAB389SP) is extremely potent ($IC_{50} = 10^{-12}$ M) and selectively destroys both native and stably transfected SPR-expressing cells in vitro (Fisher et al., 1996). Such a SPR-targeted neurotoxin may selectively lesion nociceptive neurons in vivo, and allow further study of the role of SP and the SPR in nociception. Therapy of persistent pain conditions is also possible and recently hyperalgesia caused by capsaicin injection was inhibited with an intrathecally administered saporin toxin-SP molecule (Mantyh et al., 1997). However, the model used is highly focused on capsaicin-sensitive C-fibers and may not accurately reflect a broader spectrum of action against inflammatory or chronic neuropathic pain conditions. It is essential to observe the effects of this class of agents in a wide variety of persistent pain models. In the present study we examine the effects of DAB389SP on the chronic constriction injury (CCI) model and in carageenan-induced inflammation (Bennett and Xie, 1988; Iadarola et al., 1988).

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were used in all experiments. All procedures and experimental protocols were approved by the NIDR Animal Care and Use Committee, and were in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Rats were routinely habituated pre-operatively by allowing them 5–10 min in the sensory-testing apparatus for two consecutive days.

2.2. Fusion protein

The fusion protein was prepared by replacing the native receptor-binding domain of diphtheria toxin with a sequence coding for substance-P. The molecule ended with the following amino acid sequence: A*RPKPQQFFGLM*G (SP sequence italic), (Fisher et al., 1996). The recombinant protein was expressed in *E. coli*, purified, and amidated in vitro with peptidylglycine α-amidating mono-oxygenase, which removes the C-terminal glycine and converts the carboxylic acid of methionine to an amide. The concentration of DAB389SP was assessed by radioimmunoassay, using an antibody directed at the amidated carboxy-terminal of SP. Studies of DAB389SP cytotoxicity towards cells expressing the human or rat SPR, either endogenously or after stable transfection, demonstrated high potency and selectivity (Fisher et al., 1996).

2.3. Treatments and surgical procedures

2.3.1. Anesthetics

For surgical procedures rats were routinely anesthetized with 0.3 ml of a mixture of equal volumes of ketamine (100 mg/ml) and xylazine (20 mg/ml) administered intraperitoneally. For transcardial perfusions the rats were euthanized with 500 mg/kg of pentobarbital sodium.

2.3.2. Acute inflammation

Since the rats were tested within 45 min after injection of the carageenan, no anesthetics were employed. The rats are mildly restrained by one investigator while a second investigator injects 150 μl of carageenan solution (4 mg/100 μl sterile saline) superficially into the plantar surface of the rat hindpaw. This was followed by gently massaging the injection site to evenly distribute the carageenan.

2.3.3. Chronic constriction injury

Chronic constriction injury (CCI) was performed as described originally by Bennett and Xie (1988). Following anesthetic administration, the left common sciatic nerve was exposed via a mid-thigh incision. Proximal to the sciatic trifurcation, the nerve was freed of adhering tissue for about 7 mm, and four ligatures (4–0 chromic gut) were tied loosely around it with a 1.0–1.5 mm interval between each. The ligatures were such that the nerve was barely constricted, and the circulation through the superficial epineurial vasculature was not arrested; slight movement of the ligature on the nerve was possible. The incision was closed in layers.

2.3.4. Intrathecal catheterization and infusion

Rats were placed in a stereotaxic device and the suboccipital region exposed via a midline incision. The dura over the cisterna magna was opened with a small vertical incision. Fine polyethylene tubing (PE10, Clay Adams, Parsippany, NJ), pre-filled with the appropriate solution, was gently introduced to approximately 7 cm (length premarked) which was the estimated L4/5 level. Solutions were delivered in two ways; (1) via an infusion pump (Syr-

inge Pump, Model 341B, Sage Instruments, Boston, MA) connected to a 50 μl Hamilton syringe. The pump was calibrated to deliver 50 μl of solution in approximately 12 min and (2) by an implanted osmotic mini-pump (Alza Corp. Palo Alto, CA) that delivered 0.5 μl/h over 14 days. The pump reservoir was implanted subcutaneously in the interscapular region at the same time as the infusion tubing was introduced and remained there for the duration of the experiment.

2.4. Sensory testing

Sensory tests were performed in the order they are described. The examiner was blinded as to the treatment group of each rat.

Mechano-allodynia was tested with von-Frey hairs. With the rat placed on the perforated floor of an elevated platform, the von-Frey hairs were applied in order of increasing stiffness. Each hair was applied five times at intervals of 1–4 s, to slightly different loci within the sciatic territory. The first hair to evoke at least one withdrawal response was designated the threshold, for unmanipulated rats this consistently occurred at 4.93–5.46 log-mg.

Cold-allodynia was tested by the acetone spray test, in which 200 μl of acetone was applied to the rat mid-plantar hind paw through the perforated floor (Choi et al., 1994). The duration of hind paw withdrawal evoked by the temperature decrease was timed by a stopwatch and recorded.

Mechano-hyperalgesia was assayed with the pinprick test, (Tal and Bennett, 1994). The tip of a safety pin was pushed against the mid-plantar hind paw, through the perforated floor, until the skin was dimpled but not penetrated. The duration of the pinprick evoked nociceptive withdrawal reflex was timed with a stopwatch. Normal responses consist of a very short withdrawal and are too quick to time accurately. Therefore, we defined the minimum, normal response duration as 0.5 s. Data are expressed as withdrawal duration.

Heat-hyperalgesia was assayed with a withdrawal latency test as described (Iadarola et al., 1988). Rats are placed on the glass floor of an elevated platform. A high intensity, movable radiant heat source, was placed underneath the glass and aimed at the plantar surface of one hind paw. Care was taken to stimulate the hind paw only when it was in contact with the glass floor of the test apparatus. Stimulus onset activated a timer that was controlled by a photocell. The hind paw withdrawal reflex interrupted the photocell's light and automatically stopped the timer. Latencies of the reflex were measured from the onset of radiant heat until hind paw withdrawal to the nearest 0.1 s. Each hind paw was tested five times at intervals of 5 min. The average latency of the control hind paw was subtracted from the average latency of the affected hind paw to establish the difference score. Negative difference scores indicate a lowered threshold on the affected hind paw (Bennett and Xie, 1988).

2.5. Immunocytochemistry

2.5.1. Tissue preparation and staining

Transcardial perfusion was performed by exposing the thoracic cavity and cannulating the aorta, through the left ventricle. Approximately 300 ml of ice-cold phosphate buffered saline (PBS) were pumped through the vascular system, followed by an ice-cold solution of freshly made 4% paraformaldehyde in PBS. The L_4 and L_5 dorsal root ganglia were exposed, their roots traced to their entry point into the dorsum of the spinal cord and an 8 mm portion of the lumbar enlargement including L_4–L_5 segments were removed. The spinal cord was postfixed in the same fixative overnight, and transferred to 30% sucrose in PBS for 1–3 days for cryoprotection.

Tissue sections (30 μm) were cut with a cryostat at −20°C and processed for immunocytochemistry. Sections were treated with 0.75% Triton X-100 and blocked with 3% normal goat serum (NGS). Sections were incubated overnight in 3% NGS/Triton containing SPR specific primary antiserum (1:250 000) (Vigna et al., 1994), or NMDA receptor NR1 subunit (NMDA NR1) antibody (1:2000) (Iadarola et al., 1996), or c-Fos antibody (1:3000) (Young et al., 1991). All tissues were then incubated with biotinylated goat-anti-rabbit secondary antiserum at a concentration of 1:2000 and avidin coupled to biotinylated horseradish peroxidase complex according to the manufacturers' instructions (Vector Labs., Burlingame, CA), then developed and mounted as described below. In the sections incubated with the SPR antibody, further sensitivity was attained by employing tyramide signal amplification (NEN Life Sciences Products, Boston, MA). In these sections the secondary antibody was used at a concentration of 1:4000, then the sections were washed five–eight times in PBS over 15 min, and incubated in biotinylated tyramide (1:200) in a solution of 0.005% H_2O_2, for 10 min. Following five–eight washes in PBS over 15 min, these sections were then reincubated with avidin and biotinylated HRP complex (Vector Labs Inc.). The reaction product was developed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, MO), nickel-intensified for the SPR, containing 0.01% H_2O_2 in 0.1 M phosphate buffer (pH 7.4). Sections were mounted on gelatin-coated slides, dried overnight, cleared and coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY).

2.5.2. Antisera

The c-Fos antibody has been described. It is directed against the conserved DNA binding region of the Fos family of proteins and recognizes both c-Fos, FosB, Fra1 and Fra2, thus, we are detecting Fos-immunoreactive proteins (Quinn et al., 1989). The immunogen for the NMDA NR1 C2-splice variant was to a unique peptide sequence, QYHPT-DITGPLNLSDPS, in the new C-terminus introduced by alternative splicing of exon 22 and the 3′ untranslated region (Sugihara et al., 1992). The peptide was conjugated via a C-terminal cysteine to sulfo-succinimidyl-4 (*N*-maleimido-

methyl) cyclohaxene-1-carboxylate (sulfoSMCC, Pierce, Rockford, IL) -derivatized keyhole limpet hemocyanin (Calbiochem, La Jolla, CA). Antisera were raised in two rabbits. The antiserum from one rabbit was affinity purified against the peptide immunogen immobilized on an iodoacetyl column (Sulfolink, Pierce). This antisera yields: (a) one major band of immunoreactivity at ~117 kDa on Western blot corresponding to the M_r of the NR1 subunit (b) one band with extracts of CHO cells transiently transfected with a plasmid expressing the exon 22-splice variant of the NR1 subunit, and (c) a prominent reaction product in the neuronal perikarya which allows us to count the cells. Its properties will be described more fully in a subsequent publication.

2.6. Cell counts

Cells in lamina I of the medial dorsal horn were counted in sections stained for c-Fos or NMDA NR1 immunoreactivity. Cell counts between two independent investigators were never discrepant by more than ±3. Cells were counted in three sections taken from each of three rats per group ($n = 3$).

2.7. Treatment Protocols

2.7.1. Chronic constriction injury

Experiment 1, CCI with preemptive infusion. The effects of delivering DAB389SP by slow infusion using an implanted osmotic pump followed by induction of chronic pain were examined in this experiment. Rats were implanted with a mini-pump that delivered 10^{-8} M of DAB389SP ($n = 8$) or physiological saline ($n = 8$) over a 14 day period. Following baseline testing the CCI operation was performed at 2 days post-catheter placement. Behavioral testing was performed at 5, 7, 10, 12, and 14 days post-catheter placement.

Experiment 2, established CCI treated with acute infusion. This experiment was performed in a different order to observe the effects of the DAB389SP on established chronic pain. Baseline sensory testing (von Frey, cold, pinprick and heat) was performed. Two days later the rats underwent CCI of the left common sciatic nerve. Post-operative recovery was uneventful and all rats showed hindlimb guarding by the third post-operative day. Full sensory testing was performed at the 5th and 6th post-operative days and the rats were divided into two representative groups. On the 7th post-operative day, the rats were randomly infused with 50 μl of a blindly assigned solution. The first group ($n = 11$) received sterile saline and the second group ($n = 11$) a 10^{-8} M solution of DAB389SP. Full sensory testing was performed on days 9, 11, 13, 15 and 17 post-CCI. On day 23, six rats (three from the DAB389SP treated group and three from the saline treated group) had their spinal cords removed for immunocytochemistry. The final number of rats in this experiment was: saline, ten; DAB389SP, seven. Four rats were euthanized due to motor impairments from cannula placement.

2.7.2. Acute inflammation

Experiment 3, carageenan model with pre-emptive acute infusion. Using the infusion pump, 50 μl of solution was introduced over a 12 min period. Two groups were blindly assigned to active or control treatments. The first group ($n = 9$) received sterile physiological saline, and the second group ($n = 7$) a 10^{-8} M solution of DAB389SP. A sham group was added ($n = 6$) in which all operative procedures where carried out but no catheter was introduced and no solution was injected. At the end of each procedure the wound was closed in layers. Three rats from the saline group were euthanized due to motor impairment following surgery. Seven days post-operatively all rats were weighed again (weight maintained at 275–325 g) and baseline sensory testing was performed. The final number of rats in the study was sham, six; DAB389SP, six; saline, six. Acute inflammation of the left hind paw was induced using carageenan as described. Heat and pinprick testing was performed at 45 min, 4, 8, and 24 h post-injection. Additionally, von Frey and cold tests were performed at 8 and 24 h post-injection. Following the 24 h testing, three rats from each group were euthanized and the spinal cords harvested for immunocytochemistry.

Experiment 4, carageenan model with preemptive infusion via implanted pump. Rats received DAB389SP ($n = 7$) or physiological saline ($n = 8$) that was delivered intrathecally via an implanted osmotic pump. Six days later, acute inflammation of the hind paw was induced and the rats tested at 2, 5 and 26 h after carageenan injection.

2.8. Statistics

Results were analyzed with either repeated measures or factorial analysis of variance (ANOVA) to test the omnibus null hypothesis, that there was no difference between the means in the groups. Post-hoc testing between experimental groups (pairwise comparisons) was performed with a Student Newman–Keuls procedure. Cell counts were compared using a Mann–Whitney *U*-test. In all statistical analyses, criterion for significance was $P \leq 0.05$.

3. Results

3.1. Experiment 1, CCI with preemptive infusion

In the CCI model, DAB389SP effectively prevented the development of mechano-hyperalgesia (MH) and mechano-allodynia (MA), repeated measures ANOVA for the treatments main effect: MH, $d.f. = 1$, $F = 27.4$, $P = 0.0019$; MA, $d.f. = 1$, $F = 3.21$, $P = 0.1238$. Heat-hyperalgesia was significantly reduced in DAB389SP treated rats relative to the saline treated rats (repeated measures ANOVA for

treatment main effect; $d.f. = 1$, $F = 7.464$, $P = 0.034$). Data points for MH from the CCI hind paw in the DAB389SP group approached those of the saline control hind paw at 10 and 12 days post-catheter placement, and remained low for the full 14 days (Fig. 1). MA responded in a similar fashion on days 7 and 10. At each of these time points differences between DAB389SP treated and saline treated rats were statistically significant ($P < 0.05$). No difference was observed between heat withdrawal latencies of the control

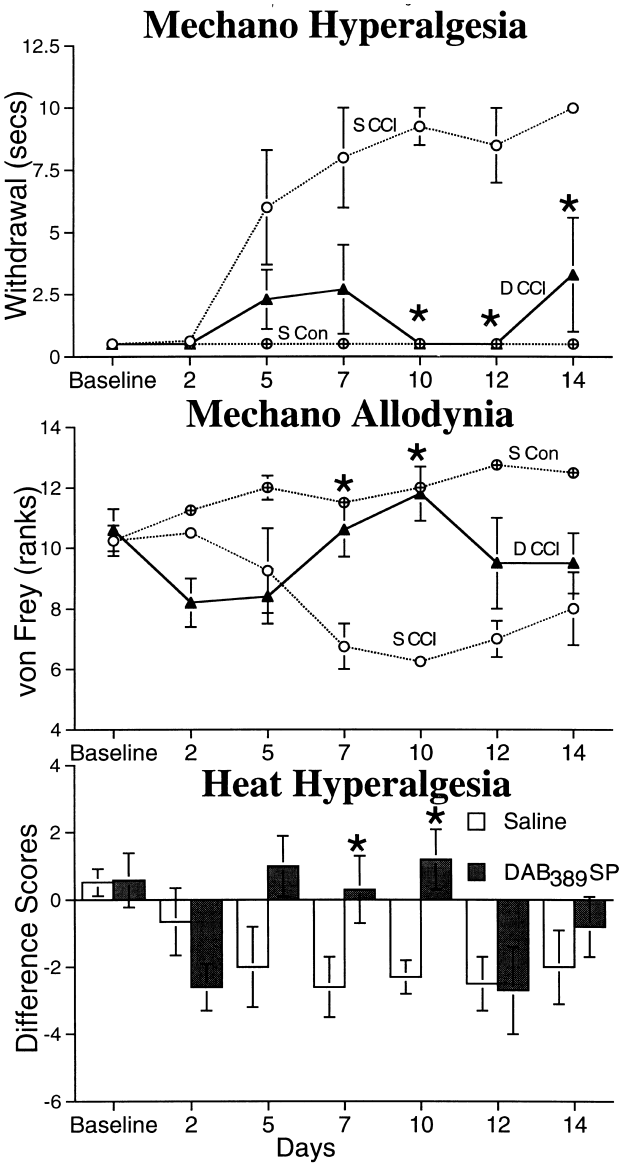

Fig. 1. The effects of pre-emptive DAB389SP on CCI as a function of days after placement of the intrathecal catheter. Significant inhibition (*) of mechano- and heat-hyperalgesia, and mechano-allodynia was observed, starting at 7 days. There was no significant difference for any of the measurements between the control hind paw of saline treated rats (shown) and the control hind paw of DAB389SP treated rats (not shown). Control hind paw heat latencies did not vary significantly from baseline in both groups (absolute values given in Section 3). Abbreviations: S CCI, saline treated rats (CCI hind paw); D CCI, DAB389SP treated rats (CCI hind paw); S Con, saline treated rats (control hind paw).

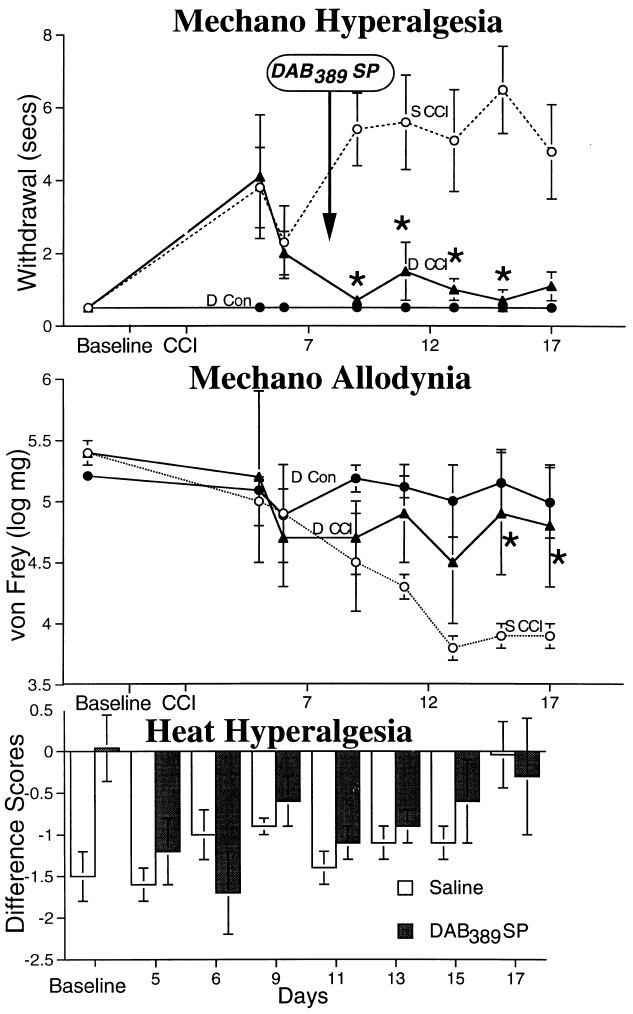

Fig. 2. The effects of acute DAB389SP infusion on established CCI as a function of days after induction of CCI. The arrow in the upper panel designates the time of DAB389SP infusion. Significant (*) reductions in mechano-hyperalgesia and mechano-allodynia were observed beginning within 2 days following DAB389SP infusion. No effect on heat-hyperalgesia was obtained in this experiment. There was no significant difference for any of the measurements between the control hind paw of DAB389SP-injected rats (shown) and the control hind paw of saline-injected rats (not shown). Control hind-paw heat latencies did not vary significantly from baseline in both groups (see Section 3). Abbreviations: S CCI, saline treated rats (CCI hind paw); D CCI, DAB389SP treated rats (CCI hind paw); D Con, DAB389SP treated rats (control hind paw).

hind paw in saline and DAB389SP treated rats, at any of the time points.

3.2. Experiment 2, established CCI treated with acute infusion

Attenuation of mechano-hyperalgesia in the DAB389SP treated rats was significant as of the second post-catheter day, and lasted for the duration of the experiment, (repeated measures ANOVA for treatment main effect; $d.f. = 1$, $F = 12.55$, $P = 0.0033$) (Fig. 2). Statistically significant reductions in mechano-hyperalgesia in the CCI hind paw between

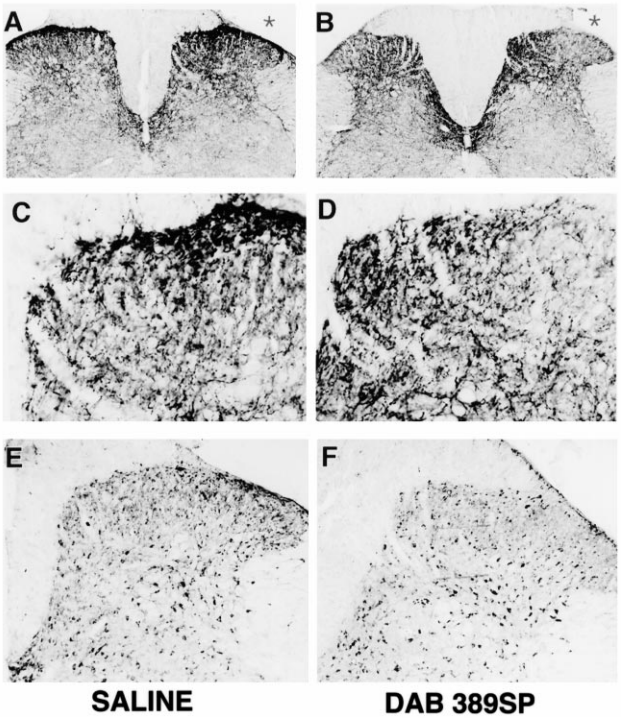

Fig. 3. Immunocytochemical staining of lumbar spinal cord sections from rats with CCI of the hind paw following acute DAB389SP administration. (A) Saline control. Intense bilateral immunoreactivity for SP receptors is seen in lamina I (CCI side indicated by *) and around the central canal (magnification ×25). (B) DAB389SP treated rats. Intrathecal administration of the toxin-SP fusion protein reduced SP receptor immunoreactivity in lamina I (magnification ×25). The effect is bilateral in the superficial dorsal horn (CCI side indicated by *) but spares the neurons around lamina X that express the SP receptor. (C) and (D), ×200 magnification of the medial dorsal horn (CCI side) of sections shown in panels A and B, respectively. (E) Section from saline treated rat (CCI side) stained for NMDA NR1 subunit showing numerous dark-staining cells. In the DAB389SP treated rat (F), NMDA NR1 subunit immunoreactivity is significantly reduced in lamina I; counts of cells in the medial dorsal horn revealed a 43% decrease (see Section 3).

DAB389SP treated and saline treated rats ($P < 0.05$) were seen on days 2, 4, 6, and 8 post-infusion. These time points correspond to 9, 11, 13, 15 days following CCI induction. The reduction in mechano-allodynia in DAB389SP treated rats was statistically significant ($P < 0.05$) relative to saline treated rats at 8 and 10 days following infusion (15 and 17 days post-CCI), (Fig. 2). In this experiment, no significant difference or trend was observed in the heat-hyperalgesia (repeated measures ANOVA for treatment main effect; $d.f. = 1$, $F = 0.337$, $P = 0.571$). At baseline, mean heat latencies for the control hind paw were 7.4 ± 0.8 s in the DAB389SP treated group, and 7.8 ± 0.4 s in the saline treated group. This similarity was maintained throughout the experiment. At 4 and 8 days following DAB389SP infusion the control hind paw latencies for the DAB389SP treated group were 6.9 ± 0.4 and 7.1 ± 0.7 s, respectively, and in the saline treated group 6.4 ± 0.3 and 7.4 ± 0.3 s, respectively. Analysis of the absolute mean heat latencies for the control hind paw in both DAB389SP treated and saline treated rats, with a factorial ANOVA, showed no significant effect for treatment at any time point.

Sections from the lumbar spinal cord stained for SP and NMDA NR1 receptors, revealed a marked staining difference between DAB389-treated and saline treated rats (Fig. 3). In saline treated rats a densely staining band of SPR immunoreactive cells was seen in lamina I, that extended medially along the gray matter to the central canal region.

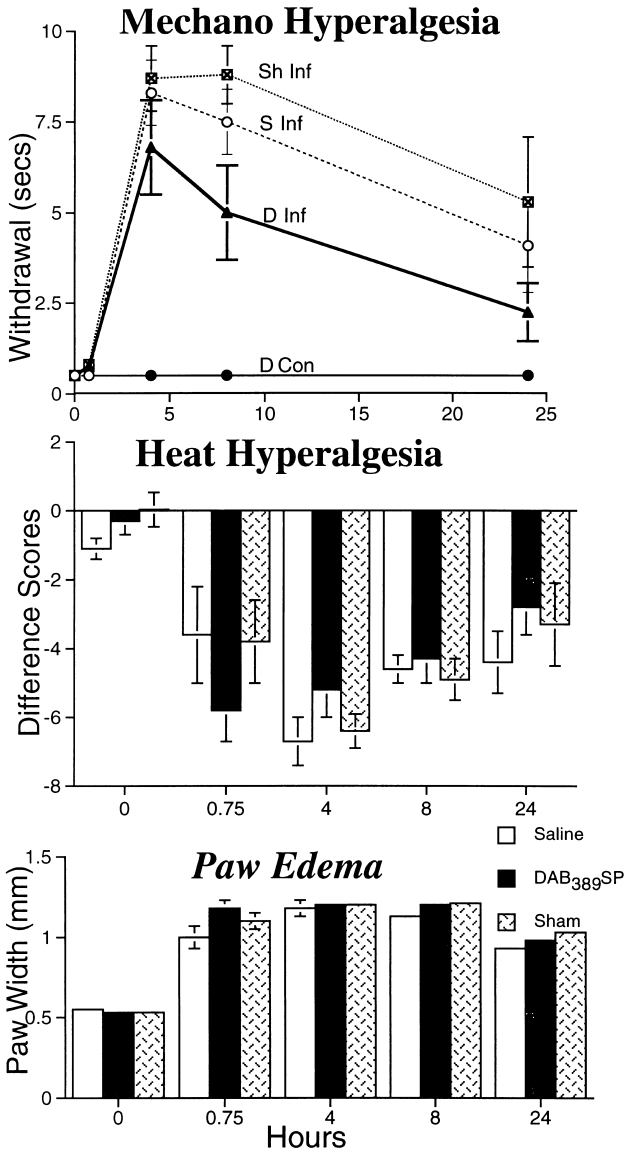

Fig. 4. The effects of DAB389SP on carageenan-induced inflammation plotted versus h from carageenan injection. Intrathecal DAB389SP had no effect on edema (i.e. did not inhibit development of the peripheral inflammation). In this experiment the attenuation of mechano-hyperalgesia approached statistical significance (see Section 3). No difference was seen for mechano-hyperalgesia in the control hind paw between any of the groups (data for control hind paw in DAB389SP group shown). DAB389SP had no effect on heat hyperalgesia and did not affect control paw heat latencies (see Section 3). Abbreviations: Sh Inf, Sham group (inflamed hind paw); S Inf, saline group (inflamed hind paw); D Inf, DAB389SP group (inflamed hind paw); D Con, DAB389SP group (control hind paw).

This staining pattern is similar to previous localization studies of SPR-expressing cells in spinal cord (Vigna et al., 1994; Brown et al., 1995). Treatment with DAB389SP caused a decrease in SPR immunoreactivity in lamina I, but the staining seen around the central canal (lamina X) remained intact. When the cells in lamina I were counted for NMDA NR1 (C2-) immunoreactivity (Fig. 3), a mean of 23.3 ± 0.9 positive cells were found per section examined from the DAB389 treated rats, and a mean of 40.7 ± 2.3 per section in the saline treated group. This represents a 43% reduction in NMDA NR1 immunoreactive cells in the DAB389SP treated group relative to the saline treated group and is statistically significant (Mann–Whitney *U*-test; *U* prime = 9, tied *P*-value = 0.05).

3.3. Experiment 3, carageenan model with pre-emptive acute infusion

A hyperalgesic response was observed in all groups following carageenan injection. No difference was noted in carageenan induced hind paw edema between the groups (Fig. 4). Rats treated with DAB389SP showed some attenuation of mechano-hyperalgesia that was most apparent at 8 h post-injection, (Fig. 4) but no statistical significance was achieved overall, as shown by the repeated measures ANOVA analysis for the treatment effect (*d.f.* = 2, $F = 2.779$, $P = 0.094$). A factorial ANOVA at the 8 h time point revealed a borderline significance (*d.f.* = 2, $F = 3.538$, $P = 0.055$), however, post-hoc testing (Student Newman–Keuls) revealed statistical significance only between the DAB389SP-treated and sham-treated groups ($P = 0.02$). There was no significant effect of DAB389SP on heat hyperalgesia. No effects were noted on control hind-paw heat latencies in any of the groups. At baseline, the mean heat withdrawal latencies for the control hind paw in the DAB389SP treated group were 7.4 ± 0.5 s, 6.6 ± 0.4 s in the saline treated group, and 7.5 ± 0.6 s in the sham-treated group. At 4 and 24 h post-inflammation control hind-paw heat latencies for the DAB389SP treated group were 9.1 ± 0.6 and 8.5 ± 0.5 s, respectively; in the saline treated group 9.3 ± 0.4 and 8.7 ± 0.5 s, respectively; and in the sham group 8.9 ± 0.7 and 8.3 ± 0.4 s, respectively. An ANOVA of the absolute mean heat latencies for the control hind paw in DAB389SP treated, saline treated and sham-treated rats showed no significant differences between these groups at any time points.

Immunocytochemical examination of the spinal cord sections from rats sacrificed 24h post-injection revealed that, relative to both sham and saline controls, the DAB389SP treated group showed a decreased SPR immunoreactivity and a decrease in inflammation-induced c-Fos immunoreactive nuclei (c-Fos Ir) (Fig. 5). Cells were counted as described (Section 2) for c-Fos Ir. A mean of 24 ± 2.6 c-Fos Ir cells were counted per section from the DAB389SP treated group and a mean of 48.8 ± 2.2 c-Fos Ir cells were counted per section from the saline treated group. This reflects an average reduction of 51% in the number of c-Fos Ir cell nuclei counted in lamina I in the sections from the DAB389SP treated group relative to the sections from the saline treated group and is statistically significant (Mann–Whitney *U*-test; *U* prime = 9, tied *P*-value = 0.05).

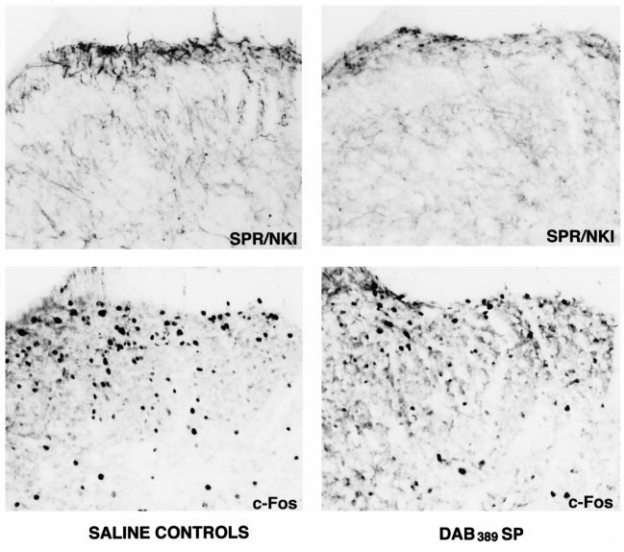

Fig. 5. Immunocytochemical staining of lumbar spinal cord sections from rats with carageenan inflammation of the hind paw. DAB389SP infusion reduced SPR immunoreactivity in DAB389SP treated, relative to saline treated rats (top panels). Similarly c-Fos expression was significantly reduced (51%) in the DAB389SP treated rats relative to the saline treated rats (lower panels).

3.4. Experiment 4, carageenan model with pre-emptive infusion via implanted pump

In this experiment no substantial effect of DAB389SP was seen in measurements of hyperalgesia. Rats treated with DAB389SP showed reduction of heat-hyperalgesia at 26 h post-carageenan injection; their average withdrawal latency was 11.8 s relative to 7.7 s in controls, with a statistically significant difference ($P < 0.05$). At earlier time points no significant difference was found. By 48 h the inflammation was resolving, reducing assay sensitivity.

4. Discussion

Our principal finding is that hyperalgesia from an experimentally induced neuropathic pain condition may be attenuated by diphtheria toxin targeted to SP receptors. To varying degrees this effect was seen in both inflammatory and neuropathic experimental models of pain, but the effects on chronic neuropathic pain were more pronounced. The major parameter of experimental pain alleviated was mechano-hyperalgesia, mechano-allodynia was also consistently reduced but to a lesser extent. At the dose used, heat-hyperalgesia responded variably and no improvement in cold-allodynia was observed in any of the experiments.

Inhibition of hyperalgesia was seen with toxin administration either before or after CCI had been established, either by acute infusion or osmotic mini-pump, with no overt signs of toxicity or motor dysfunction.

In the carageenan inflammation model, an improvement was seen in mechano-hyperalgesia in the DAB389SP treated group versus either the saline controls or the sham in the carageenan model, but was statistically significant only when compared with the sham procedure. A variable response for heat-hyperalgesia was observed with a significant but moderate effect in one experiment that was not sustained in a second experiment. We hypothesize that the sensory input resulting from carageenan injection may be of such intensity and concentrated in a narrow temporal window in comparison to CCI that this dose of DAB389SP produced an insufficient therapeutic effect. In contrast to the behavioral studies, a noticeable effect of DAB389SP was observed on inflammation-induced neurochemical parameters in the superficial dorsal horn. Inflammation per se induces an increase in both SPR expression and c-fos (Hunt et al., 1987; Draisci and Iadarola, 1989; McCarson and Krause, 1994). Treatment with toxin caused a partial reduction in SPR immunoreactivity (similar to the effect illustrated for CCI) and a 51% reduction in c-Fos immunoreactive nuclei. These findings reinforce the idea that the treatment regimens used in the inflammation studies caused a partial reduction in stimulus-induced nociceptive transmission from the inflamed hind paw. The partial reduction, coupled with the synchronous, massive stimulation of nociceptive afferents by the inflammation, may account for the lack of a substantial behavioral effect in the present series of carageenan experiments. In light of these findings, future experiments with inflammation will need to titrate treatment dosage against stimulus intensity to inhibit inflammatory hyperalgesia.

In the CCI model, tests of mechano-hyperalgesia and mechano-allodynia revealed a significant effect that occurred consistently in both experiments, regardless of when the DAB389SP was administered. We attribute the mechanism underlying the reduced hyperalgesia to the toxin-induced lesioning of second order pain processing neurons and consequent interruption of pain transmission locally and to higher levels of the neuraxis. Immunocytochemical staining for the SPR shows that DAB389SP treatment partially reduces the amount of SP receptor immunoreactivity in the dorsal horn. While the reduction occurs bilaterally, the latencies of the non-inflamed (control) hind paw consistently remained within normal limits. This indicates that basal nociception is preserved despite the bilateral lesion and suggests that the degree of cell loss obtained in these experiments selectively modulates hyperalgesia. The preservation of basal nociception may also reflect the presence of nociceptive neurons that do not express the SP receptor. In fact, Mantyh et al. (1997) estimate that the SPR-expressing cells represent a small fraction (15%) of lamina I neurons. We purposely employed a high sensitivity technique (tyramide signal amplification) to detect potential residual SPR immunoreactivity. This method coupled with nickel-diaminobenzidine gave a dense reaction product that outlined the cell membrane of the soma and dendrites consistent with previous observations (Liu et al., 1994a). In the superficial dorsal horn, individual cells were difficult to count, however, the reduction in SPR immunoreactivity in DAB389SP treated rats was clearly discernible and consistently observed. It also corresponds to other more readily quantitated endpoints such as Fos-immunoreactive nuclei and NMDA NR1 subunit positive perikarya. We examined the latter endpoint using an antibody against the exon 22- splice variant of the NR1 subunit since the immunoreactivity for this alternatively spliced form is prominently located in the cell body and allows reactive cells to be counted easily (Iadarola et al., 1996). Counts of cells in lamina I disclosed a significant, (43%) decrease in the number of NMDA NR1 immunoreactive perikarya, the same locus where the most marked reduction in SP receptors was noted. However, unless the NMDA NR1 and SP receptors highly co-localize, any cells with only the NMDA NR1 receptor would be unaffected and the pain maintained. The pattern of receptor immunoreactivity, as seen in Fig. 3, reveals substantial residual NMDA NR1 subunit-expressing cells in lamina I, consistent with the loss of SP receptors following DAB389SP treatment. This suggests at least a partial co-localization of these two receptor types on the same second-order spinal neurons.

NMDA receptors play a major role in the generation and maintenance of dorsal horn neuron hyperactivity (Haley et al., 1990) and hyperalgesia. These receptors are activated by glutamate, which is stored in and released from primary afferent terminals (Skilling et al., 1988). More recently, a presynaptic location for NMDA receptors on primary afferent terminals has been suggested (Liu et al., 1994b). Activation of these receptors causes release of SP from primary afferent terminals and activates SPR on second order neurons (Liu et al., 1997). Analysis of dorsal root ganglion transcripts by reverse-transcriptase PCR indicates that dorsal root ganglion neurons express both the NMDA NR1 subunit and SPR (Sato et al., 1993, 1996). While these multiple receptor locations may produce a complex situation, our data suggest that the second order SPR-expressing neurons are key elements in nociceptive transmission.

Diphtheria toxin is a polypeptide with an N-terminal fragment A (Gly1 to Arg193) that contains the catalytic domain, and a C-terminal fragment B (Ser194 to Ser535) that carries the transmembrane and receptor-binding domains (Pappenheimer, 1977, Sweeney and Murphy, 1995). The enzymatic activity of the diphtheria toxin causes ADP-ribosylation of elongation factor-2 (EF2), an eukaryotic enzyme involved in mRNA translation. The inactivation of EF2 inhibits cellular protein synthesis (Pappenheimer, 1977), and may lead to cell death. DAB389SP carries the catalytic and transmembrane domains of diphtheria toxin fused to SP. In this

way DAB389SP combines specific cellular targeting using SP as the native ligand, and the toxic properties of the diphtheria molecule as the mechanism to induce cell death. This chimeric fusion protein was previously shown to cause selective cellular death in cultured cells expressing the SP receptor (Fisher et al., 1996), and is extremely potent ($IC_{50} = 10^{-12}$ M). The in vitro effect occurs rapidly: [^{14}C] leucine incorporation was totally arrested in CHO cells expressing the human and rat SP receptor 19 h following exposure to DAB389SP (Fisher et al., 1996). Extrapolating from this in vitro data to predict the results of our in vivo experiments is complex due to dilution effects upon infusion and enzymatic degradation amongst other factors. It is probably not necessary for the cell to die before its function is compromised, and early behavioral effects may be attributable to metabolic dysfunction of the second order neurons in spinal cord. This continuum of cell damage, that can lead to cell death, may include aberrant responses to nociceptive input and initial structural changes in cellular morphology (e.g. dendritic withdrawal). Our immunocytochemical studies are representative of the final time point in this dynamic process. Possibly, some affected cells may eventually recover from the effects of DAB389SP and may return to normal functionality. This can explain some waning in the effects of DAB389SP seen in the experiments (e.g. Fig. 1).

In a similar fashion, a SP-saporin toxin conjugate was also used to destroy SPR expressing neurons in the striatum and the spinal cord (Mantyh et al., 1997; Wiley and Lappi, 1997). The intrathecal administration of SP-saporin toxin conjugate prevented experimental, capsaicin-induced hyperalgesia (Mantyh et al., 1997). However, diphtheria and saporin differ in some of their properties. Following endocytosis of diphtheria toxin, the transmembrane sequence is capable of modifying the endocytic vesicle allowing the toxin to enter the cytoplasm (Pappenheimer, 1977); such a mechanism for saporin has not been identified and the release into the cytoplasm may be controlled more by concentration and rate of uptake. Mantyh et al. (1997) used a higher concentration (10^{-6} M) of the SP-toxin molecule than we used in our experiments (10^{-8}–10^{-9} M) and also observed a reduction in hyperalgesia. Their immunocytochemical studies showed SPR immunoreactivity that was indicative of selective destruction of second order SP receptor-expressing neurons in the superficial dorsal horn. However, the equivalence of the tests used in that study and the present one needs further clarification. Capsaicin selectively stimulates C-fiber endings and this population may only partially overlap with the afferent fiber population activated during carageenan-induced inflammation and CCI. It is also worth noting that we used a highly sensitive amplification method for the SPR immunocytochemistry that provides a very reliable detection of residual SPR-expressing elements.

Receptor-mediated endocytosis in drug delivery offers many advantages, which include specific delivery to target cell, non-invasive targeting, and a high uptake rate (Kato and Sugiyama, 1997). The major disadvantage is uptake by non-target cells that express the same receptor. In our experiments we found no motor or behavioral changes attributable to such uptake of DAB389SP. However, the widespread presence of the SP receptor in the CNS (Mantyh et al., 1989; Hershey et al., 1991; Brown et al., 1995) and its varied physiological functions (Otsuka and Yoshioka, 1993) will require careful monitoring with more extensive behavioral tests and possibly tests of autonomic function. Some of the critical factors that influence the behavioral results are the penetration of DAB389SP, its concentration gradient, its rate of degradation in the extra cellular space and the rate of internalization. The loss of SP receptor-expressing cells occurs mainly in the superficial dorsal horn while sparing the cells around the central canal that express the SP receptor. The sparing of SPR-expressing cells in deeper laminae of the spinal cord is very similar to the extent of SP receptor loss obtained with a saporin-SP conjugate (Mantyh et al., 1997). These data indicate a limited tissue penetration of intact SP-toxin molecules and suggest that the SP portion is susceptible to enzymatic cleavage by peptidases in tissue or cerebrospinal fluid (CSF). One candidate peptidase is angiotensin-converting enzyme (ACE) which can cleave di- and tripeptides from the carboxy end of SP (Skidgel et al., 1984). ACE is highly expressed in choroid plexus and secreted into the CSF which is a rich source of ACE activity (Unger et al., 1984; Mellstrom et al., 1986). Since the carboxy end of SP is essential for receptor recognition and DAB389SP is administered into the CSF, then cleavage by ACE may be a critical determinant of the half-life of DAB389SP and its efficacy in killing SPR expressing neurons. Another factor that influences DAB389SP activity in vitro is the density of SP receptors on the cell surface. In cells expressing human SPR, an increase in receptor number from 4000 to 490 000 receptors/cell reduces the IC_{50} of DAB389SP from 1.8×10^{-11} M to 5.1×0^{-12} M (Fisher et al., 1996). The SP receptor is highly expressed in superficial dorsal horn neurons and persistent pain states produce a further increase in SP receptor expression on second order neurons (Schaffer et al., 1993; McCarson and Krause, 1994; Abbadie et al., 1996). In vivo studies demonstrate a graded increase in SP release and SP receptor internalization in response to graded noxious thermal stimuli (Allen et al., 1997). The increased rate of internalization would increase the effective dose delivered intracellularly, and increased receptor expression would increase cell susceptibility. Uptake of toxin must be robust enough to incapacitate protein synthesis before residual synthetic machinery can compensate for the loss of EF2. In the experimental pain states, all of these factors are working together to cause targeted, and rapid, endocytosis of DAB389SP. This suggests that the toxin would have a better effect in the presence of ongoing pain or less effect if there were competition for receptor binding by endogenous SP. Neither mechanism appeared to be rate limiting since the toxin seems to work equally

well when administered before or after the establishment of the nociceptive condition (e.g. CCI or inflammation).

In conclusion, beneficial results were observed, particularly in the CCI model, without observable deleterious side effects. This paves the way for further research that may ultimately progress to non-human primate testing and clinical trials. It is likely that additional advances in the field of ligand-toxin chimeric molecules will involve neuromodulators other than substance P. Therefore, a more general term for this emerging class of molecules is needed and, following the terminology utilized in oncology, we have recently proposed the general term 'noci-toxins' (Iadarola and Caudle, 1997).

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